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APPLICATION FOR LETTERS PATENT

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Title: **MAIZE MIP SYNTHASE PROMOTER**

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MAIZE MIP SYNTHASE PROMOTER

Related Application

This application claims priority from United States Provisional Patent Application Ser. No. 60/168,612, filed December 2, 1999.

Field of the Invention

The invention provides DNA sequences and constructs that are useful in genetic engineering of plants. More particularly, the invention provides an isolated DNA sequence encoding maize myo-inositol-1-phosphate synthase (MIP synthase)and novel regulatory sequences derived from the MIP synthase gene, that can be used to drive expression of a variety of nucleic acid sequences in embryo tissue of transgenic plants.

Background of the Invention

Plant genetic engineering projects require access to a variety of genetic elements that are used to regulate transgene expression. A primary example is the promoter, which regulates initiation of transcription.

A need exists for a variety of promoters for use in genetic engineering of plants. In particular, a need exists for promoters that drive expression specifically in embryo tissue.

Brief Description of the Sequences

SEQ ID NO:1 is the DNA sequence for maize MIP synthase.

SEQ ID NO:2 is the amino acid sequence for maize MIP synthase.

SEQ ID NO:3 is the DNA sequence for the embryo specific maize MIP synthase promoter.

Summary Of The Invention

The invention provides an isolated DNA molecule encoding maize MIP synthase.

In another of its aspects, the invention provides embryo specific maize MIP synthase promoters corresponding to or derived from SEQ ID NO:3.

In another of its aspects, the invention provides a DNA construct comprising, operatively linked in the 5' to 3' direction,

a) a maize MIP synthase promoter;

b) a DNA sequence of interest; and

c) a 3'UTR.

In another of its aspects, the invention provides a plasmid comprising a maize MIP synthase promoter, preferably bp 7-2064 of SEQ ID NO 3.

In another of its aspects, the invention provides a transformed plant comprising at least one plant cell that contains a DNA construct of the invention. The plant may be a monocot or dicot. Preferred plants are maize, rice, cotton and tobacco.

In another of its aspects, the invention provides seed or grain that contains a DNA construct of the invention.

Detailed Description of the Invention

The DNA sequence of interest used in constructs of the invention may be any gene that it is desired to express or down regulate in plants. Particularly useful genes are those that confer tolerance to herbicides, insects, or viruses, and genes that provide improved nutritional value or processing characteristics of the plant. Examples of suitable agronomically useful genes include the insecticidal gene from *Bacillus thuringiensis* for

conferring insect resistance and the 5'-enolpyruvyl-3'-phosphoshikimate synthase (EPSPS) gene and any variant thereof for conferring tolerance to glyphosate herbicides. As is readily understood by those skilled in the art, any agronomically important gene conferring a desired trait or producing an important protein can be used.

The 3' UTR, or 3' untranslated region, employed in constructs of the invention is one that confers efficient processing of the mRNA, maintains stability of the message and directs the addition of adenosine ribonucleotides to the 3' end of the transcribed mRNA sequence. The 3' UTR may be native with the promoter region, native with the structural gene, or may be derived from another source. A wide variety of termination regions are available that may be obtained from genes capable of expression in plant hosts, e.g., bacterial, opine, viral, and plant genes. Suitable 3' UTRs include but are, not limited to: the *per5* 3' UTR (WO98/56921), the 3' UTR of the nopaline synthase (*nos*) gene, *tmL* 3', or *acp* 3', for example.

The present invention is generally applicable to the expression of structural genes in both monocotyledonous and dicotyledonous plants. This invention is particularly suitable for any member of the monocotyledonous (monocot) plant family including, but not limited to, maize, rice, barley, oats, wheat, sorghum, rye, sugarcane, pineapple, yams, onion, banana, coconut, and dates. A preferred application of the invention is in production of transgenic maize plants. The invention is particularly applicable to the family *Graminaceae*, in particular to maize, wheat, rice, oat, barley and sorghum. Dicotyledonous species include

tobacco, tomato, sunflower, cotton, sugarbeet, potato, lettuce, melon, soybean and canola (rapeseed).

The present invention also includes DNA sequences having substantial sequence homology with the specifically disclosed regulatory sequences, such that they are able to have the disclosed effect on expression.

As used in the present application, the term "substantial sequence homology" is used to indicate that a nucleotide sequence (in the case of DNA or RNA) or an amino acid sequence (in the case of a protein or polypeptide) exhibits substantial, functional or structural equivalence with another nucleotide or amino acid sequence. Any functional or structural differences between sequences having substantial sequence homology will be *de minimis;* that is they will not affect the ability of the sequence to function as indicated in the present application. Sequences that have substantial sequence homology with the sequences disclosed herein are usually variants of the disclosed sequence, such as mutations, but may also be synthetic sequences.

In most cases, sequences having 95% homology to the sequences specifically disclosed herein will function as equivalents, and in many cases considerably less homology, for example 75% or 80%, will be acceptable. Locating the parts of these sequences that are not critical may be time consuming, but is routine and well within the skill in the art.

It is contemplated that sequences corresponding to the above noted sequences may contain one or more modifications in the sequences from the wild-type but will still render the respective elements comparable with respect to the teachings of this invention. For example, as noted above, fragments may be used. One may

incorporate modifications into the isolated sequences including the addition, deletion, or nonconservative substitution of a limited number of various nucleotides or the conservative substitution of many nucleotides. Further, the construction of such DNA molecules can employ sources which have been shown to confer enhancement of expression of heterologous genes placed under their regulatory control. Exemplary techniques for modifying oligonucleotide sequences include using polynucleotide-mediated, site-directed mutagenesis. See Zoller et al. (1984), DNA, 3:479-488; Higuchi et al. (1988), Nucl. Acids Res., 16:7351-7367, Ho et al. (1989), Gene, 77:51-59, Horton et al. (1989), Gene, 77:61; and PCR Technology: Principles and Applications for DNA Amplification, (ed.) Erlich (1989)).

Conventional technologies for introducing biological material into host cells include electroporation (see Shigekawa and Dower (1988), Biotechniques, 6:742; Miller, et al. (1988), Proc. Natl. Acad. Sci. USA, 85:856-860; and Powell, et al (1988), Appl. Environ. Microbiol., 54:655-660); direct DNA uptake mechanisms (see Mandel and Higa (1972), J. Mol. Biol., 53:159-162; Dityatkin, et al. (1972), Biochimica et Biophysica Acta, 281:319-323; Wigler, et al. (1979), Cell, 16:77; and Uchimiya, et al. (1982), In: Proc. 5th Intl. Cong. Plant Tissue and Cell Culture, A. Fujiwara (ed.), Jap. Assoc. for Plant Tissue Culture, Tokyo, pp. 507-508); fusion mechanisms (see Uchidaz, et al. (1980), In: Introduction of Macromolecules Into Viable Mammalian Cells, Baserga et al. (eds.) Wistar Symposium Series, 1:169-185); infectious agents (see Fraley, et al. (1986), CRC Crit. Rev. Plant Sci., 4:1-46); and Anderson (1984), Science, 226:401-409); microinjection mechanisms (see Crossway, et

al. (1986), Mol. Gen. Genet., 202:179-185); and high velocity projectile mechanisms (see EPO 0 405 696 to Miller, Schuchardt, Skokut and Gould, (The Dow Chemical Company)

The appropriate procedure to transform a selected host cell may be chosen in accordance with the host cell used. Based on the experience to date, there appears to be little difference in the expression of genes, once inserted into cells, attributable to the method of transformation itself. Once introduced into the plant tissue, the expression of the structural gene may be assayed in a transient expression system, or it may be determined after selection for stable integration within the plant genome.

Techniques are known for the *in vitro* culture of plant tissue, and, in a number of cases, for regeneration into whole plants. The appropriate procedure to produce mature transgenic plants may be chosen in accordance with the plant species used. Regeneration varies from species to species of plants. Efficient regeneration will depend upon the medium, on the genotype, and on the history of the culture. Once whole plants have been obtained, they can be sexually or clonally reproduced in such a manner that at least one copy of the sequence is present in the cells of the progeny. Seed from the regenerated plants can be collected for future use, and plants grown from this seed. Procedures for transferring the introduced gene from the originally transformed plant into commercially useful cultivars are known to those skilled in the art.

In one of its aspects, the invention is regarded as encompassing any deleted version of the MIP synthase promoter that provides a functional plant promoter. Such

promoters are encompassed by the term "MIP synthase promoter". A sequence will be regarded as providing a "functional" promoter for purposes of this application if it gives transient GUS expression above background levels when tested as in Example 4. Those skilled in the art will understand that various deletions from the 2058 bp sequence (bp 7-2064 of SEQ ID NO:3) can be made without destroying functionality of the sequence as a promoter. Deletion experiments are within the skill in the art. Preferably, a promoter of the invention will comprise 200 contiguous base pairs that are identical to 200 contiguous base pairs of the sequence defined by bp 7-2064 of SEQ ID NO:3. More preferable are promoters that comprise 500 contiguous base pairs that are identical to 500 contiguous base pairs of the sequence defined by bp 7-2064 of SEQ ID NO:3.

In the following examples, standard methods of DNA purification, restriction enzyme digestion, agarose gel analysis, DNA fragment isolation, ligation and transformation were used, as described in Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989); Molecular Cloning a Laboratory Manual, second edition. (Cold Spring Harbor: Cold Spring Harbor Laboratory Press), Ausubel, F.M., Brent, R., Kingston, R., Moore, D., Smith, J., Seidman, J., and Struhl, K., eds. (1987); and Current Protocols in Molecular Biology. (New York: John Wiley and Sons).

Example 1

Cloning of a maize cDNA encoding MIP Synthase

A. Isolation of a Maize MIP Synthase Probe Using Degenerate Primers

A probe was isolated by PCR amplification of maize embryo cDNA using degenerate primers designed from the yeast MIP synthase amino acid sequence. At the time only the yeast MIP synthase sequence was known (Johnson, M. and Henry, S. (1989). Biosynthesis of Inositol in Yeast: Primary Structure of Myo-Inositol-1-Phosphate Synthase (EC 5.5.1.4) and Functional Analysis of its Structural Gene, the INO1 Locus. J. Biol. Chem. 264: 1274-1283.), it was not possible to identify "conserved" regions of the MIP synthase protein sequence. As an alternative, those amino acids that are encoded by only one or two codons were identified in the yeast protein sequence. Stretches of five or more of these low redundancy amino acids were selected as regions for primer design.

A clone (MP18) that could be translated into protein that had identity with yeast MIP synthase was identified. The insert of MIP18 was gel purified, labeled with ^{32}P and used to probe a lambda maize embryo cDNA library.

B. Isolation of a Maize MIP Positive cDNAs

Protocols for phage plating, plaque purification and in vivo excisions were as recommended by the manufacturer (Stratagene, LaJolla CA). Some changes were introduced and are noted below.

E. coli XL-1 blue were grown in NZY media containing 0.2% maltose to an optical density of 1.0 at 520 nm. The

cells were collected by centrifugation at low speed and resuspended to the same density in 10 mM $MgSO_4$. Cells were stored for several days at 4°C with little loss in plaque forming efficiency. Phage were preabsorbed to 200 µL of cells for 15 minutes at room temperature in Falcon 2059 tubes followed by 15 minutes at 37°C. The cells were plated in 3 mL NZY agarose at 48°C on to NZY plates. Plates were incubated at 37°C overnight.

Plates were chilled, 0.22 micron nylon filters were gently applied to the plate and allowed to absorb phage for 2 minutes. The filters were transferred to blotting paper saturated with 0.5 M NaOH, 1.5 M NaCl for 5 minutes. The filters were allowed to dry for 5 minutes then transferred to blotting paper saturated with a neutralization solution of 0.5M Tris pH 7.6, 1.5M NaCl for 15 minutes. The filters were then cross-linked using a Stratagene UV cross-linker on the "auto" setting. The filters were washed with two changes of 2X SSC, 0.1% SDS. Prehybridization was a minimum of 6 hours in 6X SSC, 10X Denhardt's solution, 0.1% SDS, 200 mg/mL DNA at 42°C.

DNA fragments were isolated using the Qiaex purification methods of Qiagen Inc., (Chatsworth, CA). The Boehringer Mannheim Random Primed DNA Labeling Kit (Indianapolis, IN) was used following manufacturer's instructions. Unincorporated nucleotides were removed by gel filtration through a Stratagene PUSH column following manufacturer's recommendations.

Hybridization solution for low stringency hybridization was 6X SSC, 10X Denhardt's solution, 0.1% SDS, 200 mg/mL

DNA, 42°C, 6 hours. Low stringency washes were 40°C, 6X SSC, 1% SDS, 4 changes in a total of 2 liters. Hybridization solution for high stringency was as above except adjusted to 50% deionized formamide. Wash conditions were 0.1% SSC, 0.1% SDS, 60°C, 4 changes in a total of 2 liters.

The primary screen yielded many positive plaques that appeared on duplicate filters. The frequency of positive plaques approached 1% indicating that the gene was highly expressed in embryo. Several plaques were picked and screened a second and third time. Eight single plaques were picked from pure stocks and the plasmids rescued for cDNA insert analysis.

The eight MIP synthase positive clones were digested with restriction endonucleases Eco RI and Xho I to release the inserts from the vector. Based on the yeast sequence, a cDNA insert of approximately 2 kb was expected, this includes 500 amino acids of coding capacity and several hundred base pairs of nontranslated sequence. Two clones contained inserts that comigrated with the 2 kb marker. The other six clones contained inserts significantly smaller in size and were not characterized further. One clone with a cDNA insert of approximately 2 kb was chosen for DNA sequence analysis, it was called clone pMIP-7.

C. DNA Sequence Analysis of Maize MIP synthase cDNA

The DNA sequence and the deduced amino acid sequence for the maize MIP synthase is shown in SEQ ID NO:1. The cDNA is 1959 nucleotides in length. The 5′ most ATG is located at position 137 giving a putative 5' noncoding

region of 136 nucleotides. A large open reading frame extends from the ATG at position 137 to a stop codon at position 1667. The reading frame encodes a polypeptide of 510 amino acids. A stop codon is located at position 1667 followed by 248 nucleotides of 3' nontranslated region. A short poly (A) tract is located at position 1918.

Example 2

Analysis of Tissue Specific and Developmental Patterns in Seed

Mycogen proprietary maize genotypes CS608, HO1, CQ806, OQ414, and HiII, and a commonly available inbred B73, were grown under standard greenhouse conditions. For analysis of tissue specific gene expression of the MIP synthase promoter, tissues were harvested at the developmental times of interest and frozen at -70°C until RNA extraction. For determination of the temporal expression pattern of MIP in seed embryos, kernels were dissected from ears of CQ806 and HO1 at different days after pollination (DAP). Following harvest, kernels were immediately dissected, embryos were collected and frozen in 50 ml conical tubes on dry ice. RNA was extracted and prepared for northern analysis using standard techniques. A MIP synthase hybridization probe was prepared from plasmid pMIP7 (for a description see Example 1) by digestion with EcoR1 and Xho1, followed by gel purification of the approximately 1950 bp insert. Twenty-five nanogram of gel-purified fragment was labeled with 50 µCi [α-32P]-dCTP (NEN Research Products) using READY-TO-GO labeling beads (Pharmacia) according to the manufacturer and purified over NUCTRAP push columns (Stratagene). The labeled probe was denatured by boiling for 5 min, chilled on ice for 5 min, and added directly to the prehybridized blots. Hybridization was done in

SEAL-A-MEAL bags (DAZEY Corp., Industrial Airport, KA), at 42°C for 16 h. Blots were washed six times for 30 min in large excess (500 mL) of pre-warmed washing solution [20 mM sodium phosphate pH6.5, 50 mM NaCl, 1 mM EDTA, and 0.1% SDS] at 60°C. Hybridization results indicated that MIP synthase was expressed in embryo tissues from each of the maize genotypes tested. Maximum expression in the embryos was observed 18-27 DAP. No significant expression was observed in leaves or roots. These data suggested that expression of MIP synthase was preferentially regulated in embryo tissues.

Example 3

Cloning Of The 5′ Untranslated Regions from the Maize MIP Synthase Gene

The maize MIP synthase 5′ flanking sequences were isolated from maize genomic DNA, var. OQ414 (proprietary line of Agrigenetics Inc., d/b/a Mycogen Seeds). DNA sequencing was accomplished using the ABI Prism DNA Sequencing Kit with AmpliTaq® Polymerase FS as described by the manufacturer (Perkin Elmer/Applied Biosystems Division, Foster City, CA). Sequencing reactions were run on an Applied Biosystem 373A DNA sequencer (Perkin Elmer/Applied Biosystems Division). The DNA sequence for the MIP synthase promoter is given in SEQ ID NO:3.

Description of Vectors

Four expression vectors were constructed which incorporated the MIP synthase promoter upstream from the β-glucuronidase (GUS) gene on a pUC19 backbone.

pMipGP339-1 and pMipGN345-1 were designed to test GUS expression in transient assays. The difference between these two vectors was that different 3′ untranslated sequences were used as transcription terminators.

pMipGP339-1 used the *per*5 3'UTR; pMipGN345-1 used the *nos* 3'UTR.

pMipGP341 and pMipGN350-1 are derivatives of pMipGP339-1 and pMipGN345-1 that add a selectable marker gene (phosphinotricin acetyl transferase (BAR) gene of Streptomyces hygroscopicus (White et al., (1989) Nucleic Acids Res. 18:1062)) driven by a double enhanced 35S promoter. pMipGP341 and pMipGN350-1 were used to test the MIP synthase promoter/GUS fusions in stably transformed maize embryos.

Plasmid UGP232-4 was used as a positive control in the transient expression studies. UGP232-4 is similar to pMipGP339, except that the GUS gene is driven by the ubiquitin 1 (*ubi*) promoter and intron I from maize in place of the MIP synthase promoter.

Plasmid pDAB305 was used as a control in the transient expression studies to standardize GUS expression across multiple experiments. pDAB305 is similar to pMipGN345-1, but uses the double enhanced 35S promoter used in pMipGN350-1 to drive expression of the GUS gene.

Production of the GUS protein from genes controlled by different promoter versions was often compared relative to an internal control gene that produced firefly luciferase (De Wet et al. (1987). Mol. Cell. Biol. 7(2), 725-37). A plasmid (pT3/T7-1 LUC) containing the luciferase (LUC) coding region was purchased from CLONTECH (Palo Alto, CA), and the coding region was modified at its 5' and 3' ends by standard methods to permit the isolation of the intact luciferase coding region on a 1702 bp fragment following digestion by *Nco*I and *Bgl*II. This fragment was used to replace the GUS gene of plasmid pDAB305, so that the luciferase coding

region was expressed from the enhanced 35S promoter, resulting in plasmid **pDeLux**.

Example 4

Transient Testing Of Mip Synthase-Gus Constructs

A. Transient histochemical GUS expression in embryos.

Three single gene plasmids were used for testing transient expression of GUS driven by the MIP synthase promoter in maize embryos. pUGP232-4 (encoding the maize ubiquitin promoter fused to GUS with the per5 3' UTR) served as a positive control. pMipGP339-1 and pMipGN345-1 contained a MIP synthase promoter-GUS fusion with the per5 and Nos 3' ends, respectively. Immature zygotic embryos from the "Hi-II" genotype (Armstrong et al. (1991) Maize Genet. Coop. News Lett. 65:92-93) were harvested at 12, 18, and 20 days after pollination (DAP). The embryos were cultured one to two days on 15Ag10 callus initiation medium consisting of N6 salts and vitamins (Chu *et al*, (1978) *The N6 medium and its application to anther culture of cereal crops.* Proc. Symp. Plant Tissue Culture, Peking Press, 43-56), 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 25mM L-proline, 100 mg/L casein hydrolysate, 10 mg/L $AgNO_3$, 2.5 g/L GELRITE (Schweizerhall, South Plainfield, NJ), and 20 g/L sucrose, with a pH of 5.8 prior to autoclaving. Before transformation, the embryos were transferred to 15Ag10+SM medium (15Ag10 with 0.2 M sorbitol and 0.2 M mannitol) for four hours of osmotic pretreatment. For helium blasting, 12 embryos were arranged in a target area of approximately 1 cm^2 on blasting medium and covered with a 230 μm mesh stainless steel screen. Blasting medium differed from 15Ag10+SM medium in that it lacked silver nitrate, contained only 6 mM L-proline and was

solidified with 20 g/L TC agar (*Phyto*Technology Laboratories, LLC, Shawnee Mission, KS).

DNA was prepared for blasting using equal molar amounts of the GUS plasmids. A total of 70 µg of DNA, test DNA plus Bluescript™ DNA (Stratagene, La Jolla, CA) when necessary, was diluted in sterile water to a volume of 150 µL. The DNA and water were added to 30 mg of surface-sterilized 1.0 µm spherical gold particles (Bio-Rad Laboratories, Hercules, CA). The mixture was vortexed briefly (approximately 15 seconds) before adding 37 µL of 2.5 M calcium chloride and 15 µL of 0.1 M spermidine (free base). After vortexing for 30 seconds, the DNA and gold were allowed to precipitate from solution. The supernatant was removed and 1 mL of ethanol was added. The DNA/gold mixture was diluted 1:4 before use for transformation.

Helium blasting accelerated suspended DNA-coated gold particles toward and into the prepared tissue targets. The device used was an earlier prototype of that described in US Patent No. 5,141,131 which is incorporated herein by reference. Tissues were placed under a partial vacuum of 25 inches of Hg in the device chamber. DNA-coated gold particles were accelerated at each embryo target once using a helium pressure of 1500 psi, with each blast delivering 20 µL of the DNA/gold suspension. Following blasting, the embryos were transferred back to 15Ag10+SM medium and incubated in the dark at 27°C for 18-24 hours prior to GUS histochemical assay.

Embryos were subjected to histochemical GUS analysis (Jefferson (1987) Plant Mol. Biol. Rep. 5:387-405) by placing in 24-well microtiter plates containing 250-500 µL of assay buffer [0.1 M sodium phosphate, pH 8.0, 0.5mM

potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM disodium EDTA, 0.95 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide, and 0.6% (v/v) TRITON X-100] per well. A partial vacuum was drawn for 2-15 minutes prior to being incubated in the dark for 24-48 hours at 37° C.

Table 3 summarizes results of three experiments testing transient GUS expression of the MIP-synthase promoter in comparison to a maize ubiquitin control. Three to five targets (12 embryos/target) were blasted per construct in each experiment. Though not as intense as the control, the MIP synthase construct with the per5 3'UTR resulted in GUS expression in embryos harvested at 12, 18, and 20 DAP. The MIP synthase plasmid with the Nos 3'end also demonstrated GUS activity in 20 DAP embryos. In conclusion, moderate levels of transient expression were observed with the MIP synthase promoter in immature zygotic embryos of maize.

Table 3.

Transient GUS Expression of MIP Synthase-GUS Constructs in Maize Embryos

Days after Pollination	Plasmid		
	pUGP232-4	pMipGP339-1	pMipGN345-1
12	+++	+	nt
18	+++	++	nt
20	+++	+ to ++	+

nt=not tested

B.Transient quantitative GUS expression in maize regenerable callus.

Plasmids pMipGP339-1 and pMipGN345-1 were tested in regenerable maize callus for an indication of the level to which the MIP synthase promoter drives constitutive expression. A modified 35S promoter/GUS construct (pDAB305), which is highly expressed in maize, was used as a control. Expression of GUS driven by either pMipGP339-1 or pMipGN345-1 was determined as a percent of GUS driven by pDAB305.

pMipGP339-1 and pMipGN345-1 each resulted in expression 3% of pDAB305 which was statistically different from the control. In conjunction with the embryo data above, the insignificant constitutive expression strongly indicates MIP synthase as an embryo specific promoter.

Example 5

Production of Stably Transformed Maize Callus

Type II callus cultures were initiated from immature zygotic embryos of the genotype "Hi-II." (Armstrong et al, (1991) Maize Genet. Coop. Newslett., 65: 92-93). Embryos were isolated from greenhouse-grown ears from crosses between Hi-II parent A and Hi-II parent B or F_2 embryos derived from a self- or sib-pollination of a Hi-II plant. Immature embryos (1.5 to 3.5 mm) were cultured on 15Ag10 callus initiation medium as described herein. After four to six weeks callus was subcultured onto callus maintenance medium (initiation medium in which $AgNO_3$ was omitted and L-proline was reduced to 6 mM). Selection for Type II callus took place for ca. 12-16 weeks.

Plasmids pMipGN350-1 and pMipGP341 were independently transformed into embryogenic callus tissue. In preparation for helium blasting, 140 µg of plasmid DNA was precipitated onto 60 mg of alcohol-rinsed, spherical

gold particles (1.5 - 3.0 μm diameter, Aldrich Chemical Co., Inc., Milwaukee, WI) by adding 74 μL of 2.5M $CaCl_2$ H_2O and 30 μL of 0.1M spermidine (free base) to 300 μL of plasmid DNA and H_2O. The solution was immediately vortexed and the DNA-coated gold particles were allowed to settle. The resulting clear supernatant was removed and the gold particles were resuspended in 1 ml of absolute ethanol. This suspension was diluted with absolute ethanol to obtain 15 mg DNA-coated gold/mL.

Approximately 600 mg of embryogenic callus tissue was spread over the surface of Type II osmotic medium as described herein. Following a 4 hour pre-treatment, tissue was transferred to culture dishes containing blasting medium as described herein. Targets were individually blasted with DNA/gold mixture using the helium blast device described herein. Tissues were covered with a stainless steel screen (104 μm openings) and placed under a partial vacuum of 25 inches of Hg in the device chamber. The DNA-coated gold particles were further diluted 1:1 with absolute ethanol prior to blasting and were accelerated at the callus targets four times using a helium pressure of 1500 psi, with each blast delivering 20 μL of the DNA/gold suspension. The targets were rotated 180° after each blast. The tissue was also mixed halfway through the procedure to expose unblasted callus. Immediately post-blasting, the tissue was transferred back to Type II osmotic medium for a 16-24 h recovery period. Afterwards, the tissue was divided into small pieces and transferred to selection medium (maintenance medium lacking casein hydrolysate and L-proline but containing 30 mg/L BASTA® (AgrEvo, Berlin, Germany)). Every four weeks for three months, tissue pieces were non-selectively transferred to fresh

selection medium. After 9 weeks and up to 21 weeks in selection, callus sectors found proliferating against a background of growth-inhibited tissue were removed and isolated. The resulting BASTA®-resistant tissue was subcultured biweekly onto fresh selection medium.

Example 6

Development of Mature Somatic Embryos and Regeneration of Transgenic Plants

From these stably transformed cultures, somatic embryos were induced to develop as seed embryos by growing embryogenic callus on Murashige and Skoog basal medium, hereinafter MS medium (Murashige and Skoog, Physiol. Plant. (1962) 15: 473-497) containing 60 g/L sucrose. The callus was grown for seven days, and then somatic embryos were individually transferred to MS medium containing 60 g/L sucrose and 10μM abscisic acid, hereinafter ABA, for an additional 7 days. After 14 days of maturation, somatic embryos from different transgenic lines were assayed for histochemical expression of the GUS gene by placing in approximately 400 μL of GUS solution as described herein except without drawing a vacuum. GUS-expressing lines and non-GUS-expressing lines were identified and transferred to regeneration media. Regeneration was initiated by transferring embryogenic callus tissue to cytokinin-based induction medium, MS medium containing 30 g/L sucrose, 100 mg/L *myo*-inositol, 30 g/L mannitol, 5 mg/L 6-benzylaminopurine, hereinafter BAP, 0.025 mg/L 2,4-D, 30 mg/L BASTA®, and 2.5 g/L GELRITE at pH 5.7. The cultures were placed in low light (125 ft-candles) for one week followed by one week in high light (325 ft-candles). Following a two-week induction period, tissue was non-

selectively transferred to hormone-free regeneration medium, which was identical to the induction medium except that it lacked 2,4-D and BAP, and was kept in high light. Small (3-5 cm) plantlets were removed and placed in 150x25 mm culture tubes containing Schenk and Hildebrandt salts and vitamins, hereinafter SH medium (Schenk and Hildebrandt, (1972) Can. J. Bot. 50:199-204), 10 g/L sucrose, 100 mg/L myo-inositol, and 2.5 g/L GELRITE, pH 5.8). At least one individual plantlet from each regenerable line was sacrificed for histochemical GUS assay. Intact plantlets (3-10 cm) were placed in 15 mL conical centrifuge tubes and submersed in approximately 5-10 mL GUS assay buffer and incubated as described herein. Non-assayed plantlets were transferred to 12 cm round pots containing approximately 0.25 kg of METRO-MIX 360 (The Scotts Co. Marysville, OH) in the greenhouse as soon as they exhibited growth and developed a sufficient root system. They were grown with a 16 h photoperiod supplemented by a combination of high pressure sodium and metal halide lamps, and were watered as needed with a combination of three independent Peters Excel fertilizer formulations (Grace-Sierra Horticultural Products Company, Milpitas, CA). At the 6-8 leaf stage, plants were transplanted to five gallon pots containing approximately 4 kg METRO-MIX 360, and grown to maturity.

Primary regenerants were outcrossed with the elite inbred, OQ414. R_1 seed was collected approximately 6 weeks post-pollination.

A total of 312 Type II callus targets were blasted with pMipGN350-1 and pMipGP341. Thirty-six Basta®-resistant callus isolates were recovered from selection, however, only 29 were induced to form mature somatic embryos as

described herein. Twenty-four of these events produced some level of blue staining following histochemical GUS assay, as described herein, ranging from very faint blue to a deep indigo blue. Thirteen of these expressers plus one maize ubiquitin/GUS/Nos positive control and one (non-GUS) transgenic negative control were regenerated. Approximately 16 R_0 plants were regenerated from each of these lines. Ten of the 13 MIP regenerants produced R_1 seed.

Example 7

Gus Analysis Of Transgenic Plants

A. GUS analysis of embryos.

Embryos from pMipGP341-06.06, pMipGN350-05.01, pMipGN350-14.01, 1817-02.11 (transgenic negative control), Whisker-12.12 and Whisker-12.14 (maize Ubiquitin-GUS positive controls) were harvested 10 through 30 days after pollination (DAP) at 5 day intervals. Up to 10 kernels were collected per ear at each harvest depending on seed set of the Ro plants. According to the method of Jefferson (1987) Plant Mol. Biol. Rep. 5:387-405 as described herein, embryos were histochemically examined for GUS expression.

No GUS expression was observed in embryos of the trangenic negative control (1817-02.11). Unexpectedly, GUS was not detected in embryos of the Ubiquitin positive controls (Whisker-12.12 and Whisker-12.14). The growth of these plants was stunted and seed set was poor. However, each of the three MIP synthase events demonstrated GUS expression in the R_1 embryos. In pMipGP341-06.06, expression was observed as early as 10 DAP. For the p350 events, GUS was detected first at 15 DAP. Expression in all lines continued through maturity

at 30 DAP. Segregation generally followed Mendelian inheritance patterns.

B. GUS analysis of roots and leaves.

Two plants per transgenic event, as well as two nontransformed controls (OQ414), transformed negative controls (1817-02 plants), and positive controls expressing maize Ubiquitin-driven GUS (Whisker-12 events) were sacrificed at different developmental stages. One plant per event at the V6 (6 leaf) and VT (emerging tassel) stages was harvested and the leaves and roots were separately pooled for analysis. Additionally, the sixth leaf of several plants per event was collected at the V6 stage and individually evaluated for GUS expression.

No GUS activity was detected in the leaves or roots of the nontransformed (OQ414) or transformed negative (1817-02) controls. Variable, yet significant, GUS expression was observed in the positive control (Whisker-12 event, seven plants), ranging from 0.45 to 2.34 ng GUS equivalent/μg protein in the leaves and 0.28 to 0.47 in the roots. The MIP synthase-GUS transgenic events demonstrated no significant GUS activity in leaves or roots, leading to the conclusion that the MIP synthase promoter is embryo specific.